Application of Ultrasonography to Non-invasive Sexing Based on the Sexual Dimorphism in Gonads of Immature Barfin Flounder *Verasper moseri*

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An ultrasonographic technique for non-invasive sexing of barfin flounder *Verasper moseri* was established based on sexual dimorphism of gonadal appearance. We used here a high frequency transducer (10 MHz) attaching to ultrasonography to improve echoic images. Although gonads of 23-month-old males were impossible to discern, those of females were visible as bright oval contours in the ultrasonographic images. Accurate identification of sex was accomplished in 63 fish ranged from 204 to 274 mm in total length of 14-month-old fish, 288 to 401 mm of 19-month-old fish and 252 to 370 mm of 23-month-old fish, except for three false diagnosis. On the other hand, it was difficult to determine sex in 11-month-old fish, 98–135 mm in total length, by this technique. The present results indicate the practical usefulness of ultrasonography for a rapid, non-invasive and realtime sexing of immature flounders.

Key words: ultrasound, ultrasonography, sexing, flounder, teleost

Barfin flounder *Verasper moseri* is a large flat fish inhabiting cold sea basins around east Hokkaido. This species is promising for aquaculture and resources enhancement in northern Japan due to its high commercial value. Available methods for non-invasive sexing are desired for keeping the large adult barfin flounders rationally in limited numbers of aquaria of fry-producing facilities. Identification of sex in an early stage and management of the sex ratio in the rearing fish are also important to economize the costs for culturing the candidates of spawning adults. The sex ratio of juvenile barfin flounder easily incline toward male-rich by rearing environment, especially affected by high water temperature, during the period of sex differentiation.*⁵

At present, only a few available methods for sexing have been developed. Among them, immunological detections of maturing female-specific proteins, *e.g.* vitellogenin^{1–6)} and choriogenin^{7–9)} in sera or surface mucous, are thought to be potent practical methods for identifying maturing females. However, they are limited in application to only sub-adult or adult fish and it takes more than a few hours to get the results by these methods. On the other hand, an ultrasonographic method has been introduced as a real-time and non-invasive sexing for sub-adult Atlantic salmon *Salmo salar*¹⁰⁾ and mature adult coho salmon *Oncorhynchus kisutch*¹¹⁾ on the basis of morphological differences of gonads between sexes.

Flat fish including barfin flounder show definite sexual

dimorphism in external shape of gonads from immature stage. This characteristic enhances the usefulness of ultrasonography for sexing of flounder by imaging non-deleteriously the gonadal shape. In the present study, we established a method to identify sex in immature barfin flounder using a high-resolution ultrasonography. The size dependence limit for correct diagnosis is also regarded by examining different sizes of fish.

Materials and Methods

Fish

Immature barfin flounders used in the present study were kept at the following three facilities. (1) 16 individuals of 19-month-old and 20 individuals of 23-month-old barfin flounders, 252–370 mm and 252–370 mm in total length, respectively, were from Akkeshi Station of Japan Sea Farming Association. (2) 30 individuals of 14-month-old barfin flounders, 204–274 mm in total length, were from Iwate Fisheries Technology Center. (3) 32 individuals of 11-month-old barfin flounders, 98–135 mm in total length, were from Hokkaido Institute of Mariculture.

Instrument

An ultrasonic Echo Camera SSD-1000 (Aloka Inc.*⁶) with a 10 MHz annular array transducer, ASU-36WL-10, was used to scan immature barfin flounders. Transducers at a relatively high frequency promise high resolution but

*⁵ R. Goto *et al.*: Abst. Metg. Japan. Soc. Fisheries Sci., April, 1996, p. 70 (in Japanese).

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restricted depth of penetration and are used for clinical diagnosis of superficial or sub-surface tissues. The annular array transducer generate a cross-section image of the interfaced object 38 mm in width at the surface and 57 mm at the depth of 25 mm on a display.

Experiments

The ultrasonographic observation was performed by means of the following two experiments. In both experiments, fish were placed on the bottom of a shallow plastic tray covered by a foam rubber plate to eliminate reflection of scattered ultrasound. They were scanned by attaching the transducer head directly on the skin of fish without any ultrasonic coupling gel. Scans were made transversely at various positions of the abdominal sides moving laterally.

Experiment 1 was carried out to ascertain the non-invasiveness of the ultrasonographic scanning using live 19-month-old barfin flounders without anesthesia at Akkeshi Station of Japan Sea Farming Association. After judging gender from the ultrasonographic images, fish were implanted with radio identification tags, HS5105L (Denson/IDI, Inc) and kept in a 50 k/ aquarium for one week. Then, the fish were killed and total body length was measured, and then dissected to confirm the judgement. The ovary length was also measured in the case of females.

Fig. 1. A schematic drawing demonstrating morphological and positional differences of gonads between sexes in 23-month-old barfin flounders.

Gonads are indicated as dark shades. Rectangles reveal the scanning positions of ultrasonographic observations. OL: ovary length.

Experiment 2 was carried out to examine the size dependence limit for correct diagnosis using three groups (11-, 14-, and 23-month-old) of different sized barfin flounders. In this experiment, we used dead fish for the experiment because of the convenience of transportation from different facilities. After ultrasonographic diagnosis, fish were treated the same as in experiment 1.

Results

Ultrasonographic Observation of Gonads of Barfin Flounder

Figure 1 shows schematic drawings demonstrating morphological differences of gonads between sexes and the scanning positions of ultrasonographic observations of both sexes of 23-month-old immature barfin flounders. Ovary of the fish extends into the secondary coelom located between hemal spines of caudal vertebrae and abdominal muscle as in the other flat fish. On the other hand, immature testis along the first pterygiophore of anal fin, which lines the rear end of the coelomic cavity, never extends into the secondary coelom.

Next, the typical ultrasonographic images of the transverse sections of abdominal trunks of both sexes at the position just after the first pterygiophore of anal fin are presented in Fig. 2. In females, a pair of clear oval contours with slight internal hyperechoic area were observed attached to a bright image of the hemal spine of caudal vertebrae (Fig. 2A). The oval contours appeared beneath the hypoechoic area of the abdominal muscle and were easily distinguishable from bright contours of interhemal spine and motive muscles of anal fin. On the contrary, no oval contours were observed in males (Fig. 2B). The position, length, diameter and depth from the surface of the oval contours in females coincided well with the anatomical data of the ovaries measured after dissection. The oval contour was thus suggested to be the echoic image of the surface of ovary.

Experiment 1

On the basis of the above criterion, trials of ultrasono-

Fig. 2. Ultrasonographic images of abdominal transverse section from 23-month-old female (A) and male (B) barfin flounders. A pair of bright oval contours of ovaries (arrows) are seen in the image from the female.

Table 1. Result of ultrasonographic sexing in live 19-month-old barfin flounders

Sex	No. used	Total length (mm) mean $\pm$ SD	Body weight (g) mean $\pm$ SD	Gonad weight (g) mean $\pm$ SD	Ovary length (mm) mean $\pm$ SD	One week after Condition	Diagnostic accuracy
Female	6	354 $\pm$ 32	711 $\pm$ 235	2.88 $\pm$ 1.13	40 $\pm$ 3	live	100%
Male	10	346 $\pm$ 32	638 $\pm$ 195	0.14 $\pm$ 0.07		live	80%

Table 2. Result of ultrasonographic sexing in immature barfin flounders

Group	No. used	Total length (mm)		Ovary length (mm)		Diagnostic accuracy
		mean $\pm$ SD	range	mean $\pm$ SD	range	
23-month-old	20	297 $\pm$ 30	252–370			100%
Female	11	295 $\pm$ 17	262–319	33 $\pm$ 4	28–42	
Male	9	300 $\pm$ 41	252–370			
14-month-old	30	235 $\pm$ 17	204–274			97%
Female	20	242 $\pm$ 15	218–274	23 $\pm$ 4	15–30	
Male	10	222 $\pm$ 14	204–242			
11-month-old	32	115 $\pm$ 10	98–135			—
Female	6	115 $\pm$ 14	98–134	7 $\pm$ 1	5–8	
Male	26	115 $\pm$ 9	102–135			

graphic sexing were carried out using live 19-month-old fish. Instead of anesthesia, the eyes of the fish were covered with a wet towel paper to keep them still. It took within two minutes to judge the gender by the ultrasonographic images. After one week, there seemed to be no injury in all the fish and ascertained the non-deleteriousness of the ultrasonographic scanning. The result of the experiment is listed in Table 1. The gender of 88% individuals was correctly detected following the above mentioned criterion, however, two males were falsely diagnosed. The failure may likely be caused by an accumulated fat layer, which also yields a bright contour, at the same position to the ovary.

Experiment 2

The size dependence limit for correct diagnosis was evaluated using the three different size groups (Table 2). In 23-month-old and 14-month-old fish, the gender of 100% and 97% of individuals, respectively, was correctly detected. Their ovarian lengths were 28–42 mm and 15–30 mm, respectively. The false diagnosis of a 14-month-old female seemed likely to be due to an undergrowth of its ovaries (ovary length: 16 mm). Meanwhile, in 11-month-old juveniles with 5–8 mm ovaries, we could not detect the oval contours in any individuals including females by ultrasonography.

Discussion

The present study demonstrates the potential of ultrasonography to identify the sex of immature barfin flounder by the use of a high frequency transducer. Gonads of flat fish reveal distinct morphological differences between sexes, size, appearance and position, even in juvenile stage. In 11-month-old barfin flounder, although the testis did not develop sufficiently for identification using the naked eye, the ovary already started elongation toward the caudal fin and grew to 4.8–6.0% of the total body length (Table 2). The basis of existence

(female) or absence (male) of the gonads behind the coelomic cavity would be a particular advantage for correct gender determination ultrasonographically. Moreover, the flat body and sub-surface localization of ovary in flounders permit us to use a high-frequency transducer which is pointed out to give a better resolution.^{10,11} Ovaries were visualized as bright oval contours in 23-, 19-, and 14-month-old fish with ovary length more than 15 mm. The failure of recognition of an echoic image of ovary in 11-month-old juveniles indicates that the sizes of the ovaries are less than the limit of ultrasonographic sexing. Combining the result of the other two groups, the size dependent limit of this technique was suggested to exist around 20 cm in total length and 15 mm in ovary length.

Sexual dimorphism in genomic, morphological, physiological and endocrinological levels allow possibilities for identifying genomic or phenotypic sex. For example, identification of sex chromosomes¹² and sex specific genes^{13,14} are applicable to detect the genomic sex in some fish having genomic dimorphism. Observation of morphological differences between sexes, *e.g.* adipose fin of Atlantic salmon,¹⁵ also would be a potent method for sexing in the case that the species reveal differences in external appearance. At present, immunological detection or measurement of sex specific proteins, vitellogenin¹⁻⁶ and sex steroids¹⁶ are proposed as available procedures for practical sexing of fish. However, the techniques require some invasive treatments, puncture for blood collection, or wipe for mucous collection, and marking for identification of individual. In addition they need time from three hours to one day and restrict the maturity of fish after beginning of vitellogenesis. The present ultrasonographic technique requires only light anesthesia if any and allows real time sorting. Actually, it took less than a few minutes per individual to complete diagnosis. Barfin flounder primarily spawn at three or four years of age in captivity. Therefore, the present technique identifies sex about two or three years retroactive to their first spawning and one to two years earlier than the sexing techniques of vitellogenin and sex

steroid measurements.

Early identification of sex in young fish would permit fry-production facilities to keep candidates of broodstock under appropriate sex ratio and allows more rational management with substantial saving of costs. The present ultrasonographic technique using high frequency transducer gives rise to the earliest sexing in flounders at present. Combination of this technique with high-growth rate culture after the period of sex differentiation would provide earlier sex determination in flounders under one year of age.

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